

HBV/HCV VIRUS-LIKE PARTICLE

CROSS-REFERENCE TO RELATED APPLICATION

This application is related to provisional patent application serial no. 60/167,224, filed November 24, 1999, from which priority is claimed under 35 USC §119(e)(1) and
5 which is incorporated herein by reference in its entirety.

TECHNICAL FIELD OF THE INVENTION

The invention is related to the area of recombinant vaccines. It is particularly related to the field of chimeric antigens and virus-like particles for use in vaccines, especially combination vaccines for Hepatitis B virus (HBV) and Hepatitis C virus
10 (HCV).

BACKGROUND OF THE INVENTION

Hepatitis C virus (HCV) infects approximately 1% of the world's population and causes serious health problems. Over 75% of acutely infected individuals eventually progress to a chronic carrier state that can result in cirrhosis, liver failure, and
15 hepatocellular carcinoma. A very small fraction of chronically infected patients clear HCV naturally and resolve chronic hepatitis. *See* Alter *et al.* (1992) N. Engl. J. Med. 327:1899-1905; Resnick and Koff. (1993) Arch. Intern. Med. 153:1672-1677; Seeff (1995) Gastrointest. Dis. 6:20-27; Tong *et al.* (1995) N. Engl. J. Med. 332:1463-1466. Immunization against E2 glycoproteins of some flaviviruses (*see e.g.*, Konishi *et al.*,
20 (1992) Virology 188: 714-720), including HCV (Ishii *et al.*, (1998) Hepatology 28: 1117-1120), may protect against infection. However, attempts to express recombinant HCV E1 and E2 glycoproteins have been frustrated by the fact that these proteins are not secreted from the host cell but are retained within the endoplasmic reticulum (Dubuisson *et al.* (1994) J. Virology 68: 6147-6160).

25 One approach to making vaccines for HCV and other viruses which has been attempted is to prepare chimeric antigens consisting of fusions of hepatitis B virus surface

antigen (HBsAg) with a heterologous antigen, for example a portion of an HCV protein. *See, e.g.*, Inchauspe et al. (1998) Dev. Biol. Stand. 92: 162-168; Nakano et al. (1997) J. Virol. (1997) 71: 7101-7109; and Inchauspe et al. (1997) Vaccine 15: 853-856. The use of HBsAg is attractive for the production of immunogenic compositions such as vaccines because HBsAg is highly immunogenic and is secreted from cultured cells in the form of virus-like particles (U.S. Patent 5,098,704). Attempts to introduce small portions of viral proteins into HBsAg have succeeded in the production of virus-like particles (*see e.g.*, Delpeyroux et al. (1990) J. Virology 64: 6090-6100, who inserted an 11 amino acid segment of polio virus capsid protein into HBsAg). However, in one study only two out of six fusion proteins containing HBsAg combined with different hydrophilic domains of HCV E2 were secreted into the culture medium as virus-like particles (Lee et al. (1996) J. Med. Virol. 50: 145-151), possibly because the E2 inserts were too large or hydrophilic. The insertion site of heterologous epitopes into HBsAg may be an important factor. A study which inserted an epitope of HBV nucleocapsid (HBcAg) at various positions into HBsAg found that insertion into an internal site in HBsAg resulted in a chimeric protein that was immunogenic for HBcAg, while insertion at the C-terminus was weakly immunogenic (Schodel et al. (1992) J. Virology 66: 106-114). Insertion at the N-terminus prevented surface access of the HBcAg epitope in the resultant particles and was non-immunogenic (*Id.*). Apparently, the molecular context in which an epitope is presented is important in determining immunogenicity, probably because of subtle alterations of protein secondary and tertiary structure. This principle was further illustrated by Eckhart et al. ((1996) J. Gen. Virol. 77: 2001-2008), who introduced a conserved, six amino acid epitope of HIV-1 gp41 protein into influenza hemagglutinin and obtained neutralizing antibodies, but could not generate neutralizing antibodies when the same epitope was inserted into HBsAg. Smaller isolated epitopes are more likely to be sensitive to such effects than larger portions of an immunogenic protein.

Currently there is no method available for expressing entire E1 or E2 glycoproteins of HCV in virus-like particles for use in immunization. Available methods limit chimeric proteins based on HBsAg to the insertion of only small isolated domains of E2, which may or may not have a native immunogenic structure. Thus, there remains a

need in the art for methods and materials that can be used to express HCV antigens in an immunogenic form in virus-like particles.

SUMMARY OF THE INVENTION

5 It is an object of the invention to provide HBV/HCV chimeric antigens for use in immunogenic compositions. It is a further object of the invention to provide virus-like particles comprising HBV/HCV chimeric antigens and methods and materials for producing such virus-like particles. It is another object of the invention to provide HBV/HCV combination vaccines. These and other objects of the invention are provided by one or more of the embodiments described below.

10 One embodiment of the invention provides a virus-like particle for use as an immunogen or as a component of a vaccine. The virus-like particle comprises a first HBsAg (hepatitis B virus surface antigen) and a chimeric antigen. The chimeric antigen comprises a second HBsAg which is linked to an HCV immunogenic polypeptide. The first and the second HBsAg each comprise a substantially complete S domain.

15 Another embodiment of the invention provides another virus-like particle for use as an immunogen or as a component of a vaccine. The virus-like particle comprises a first HBsAg and first and second chimeric antigens. The first chimeric antigen comprises a second HBsAg which is linked to a first immunogenic polypeptide comprising an HCV E1 glycoprotein or a fragment thereof. The second chimeric antigen comprises a third
20 HBsAg which is linked to a second immunogenic polypeptide comprising an HCV E2 glycoprotein or a fragment thereof. The first, second, and third HBsAg each comprise a substantially complete S domain.

Still another embodiment of the invention provides fusion proteins comprising a substantially complete S domain of HBsAg and a polypeptide. In one fusion protein, the
25 polypeptide comprises (a) amino acid residues 192 to 330 of an HCV-1 polypeptide, or (b) the corresponding residues of other HCV isolates, or (c) an immunogenic sequence having at least about 80% sequence identity to (a) or (b). In another fusion protein, the polypeptide comprises (a) amino acid residues 384 to 661 of an HCV-1 polypeptide, or

(b) the corresponding residues of other HCV isolates, or (c) an immunogenic sequence having at least about 80% sequence identity to (a) or (b).

Yet another embodiment of the invention provides nucleic acid molecules which encode fusion proteins comprising a substantially complete S domain of HBsAg and a polypeptide. In one fusion protein, the polypeptide comprises (a) amino acid residues 192 to 330 of an HCV-1 polyprotein, or (b) the corresponding residues of other HCV isolates, or (c) an immunogenic sequence having at least about 80% sequence identity to (a) or (b). In another fusion protein, the polypeptide comprises (a) amino acid residues 384 to 661 of an HCV-1 polyprotein, or (b) the corresponding residues of other HCV isolates, or (c) an immunogenic sequence having at least about 80% sequence identity to (a) or (b). These nucleic acid molecules are employed as components of immunogenic compositions, which are additional embodiments.

Another embodiment of the invention provides vectors comprising nucleic acid molecules which encode fusion proteins. The fusion proteins comprise a substantially complete S domain of HBsAg and a polypeptide comprising an immunogenic fragment of an HCV-1 polyprotein.

A further embodiment of the invention provides a method of producing virus-like particles. A cell is cultured in a culture medium, whereby the cell expresses virus-like particles comprising a first HBsAg and a chimeric antigen. The chimeric antigen comprises a second HBsAg which is linked to an HCV immunogenic polypeptide. The first and the second HBsAg each comprise a substantially complete S domain. The virus-like particles are then isolated from the culture medium.

Yet another embodiment is a method of producing a cell line that expresses virus-like particles. A cell is transfected with a vector that expresses virus-like particles comprising a first HBsAg and a chimeric antigen. The chimeric antigen comprises a second HBsAg which is linked to an HCV immunogenic polypeptide. The first and the second HBsAg each comprise a substantially complete S domain. The cell is cultured to produce a cell line that expresses the virus-like particles.

Still other embodiments of the invention are cell lines that express virus-like particles. In one cell line, the virus-like particles comprise a first HBsAg and a chimeric

antigen. The chimeric antigen comprises a second HBsAg which is linked to an HCV immunogenic polypeptide. The first and the second HBsAg each comprise a substantially complete S domain. In another type of cell line, the virus-like particles comprise a first HBsAg and first and second chimeric antigens. The first chimeric antigen comprises a second HBsAg which is linked to a first immunogenic polypeptide comprising an HCV E1 glycoprotein or a fragment thereof, and the second chimeric antigen comprises a third HBsAg which is linked to a second immunogenic polypeptide comprising an HCV E2 glycoprotein or a fragment thereof. The first, second, and third HBsAg each comprise a substantially complete S domain.

10 The invention thus provides the art with novel methods and materials for the production of HBV/HCV combination vaccines.

BRIEF DESCRIPTION OF THE DRAWINGS

Figures 1A and 1B-1F show the expression vector pCMVII (Fig. 1A) and its nucleotide sequence (Figs. 1B-1F; SEQ ID NO:1).

15 Figures 2A and 2B-2I show the expression vector pCMVII-pS2-sAg (Fig. 2A) and its nucleotide sequence (Figs. 2B-2I and SEQ ID NO:2; preS2 coding sequence begins at nucleotide position 1988 and ends at nucleotide base 2152, and SAg coding sequence begins at base 2153 and ends at 2830). The amino acid sequence of the encoded preS2-S polypeptide is also displayed in Figs. 2B-2I and in SEQ ID NO:3.

20 Figures 3A and 3B-3I show the expression vector pCMVII opti 330 E1/sAg (Fig. 3A) and its nucleotide sequence (Figs. 3B-3I and SEQ ID NO:4; 330 E1 coding sequence begins at nucleotide position 1992 and ends at nucleotide base 2483 and SAg coding sequence begins at base 2484). The amino acid sequence of the encoded chimeric antigen polypeptide is also displayed in Figs. 3B-3I and in SEQ ID NO:5.

25 Figures 4A and 4B-4F show the expression vector pCMV-II-E2661-sAg (Fig. 4A) and its nucleotide sequence (Figs. 4B-4F and SEQ ID NO:6; 661 E2 coding sequence begins at nucleotide position 1997 and ends at nucleotide base 2900, and sAg coding sequence begins at base 2907). The amino acid sequence of the encoded chimeric antigen polypeptide is also displayed in Figs. 4B-4F and in SEQ ID NO:7.

Figure 5 shows the results of sucrose density gradient centrifugation of purified recombinant HBsAg virus-like particles. The experiment is described in Example 1.

Figures 6A and 6B illustrate the expression of HCVE2-HBsAg fusion protein in COS7 cells. Increasing amounts of sAg-encoding plasmid were expressed together with
5 HCVE2-sAg fusion plasmid. The experiment is described in Example 2. The capture antibody was MAb sAg, and the detecting antibody was MAb E2 (Fig. 6A) or MAb sAg (Fig. 6B).

Figures 7A and 7B show the sedimentation profile of virus-like particles expressed from COS7 cells transiently transfected with different ratios of sAg:E2-sAg
10 fusion protein. The experiment is described in Example 2. The capture antibody was MAb sAg, and the detecting antibody was MAb E2 (Fig. 7A) or MAb sAg (Fig. 7B).

Figures 8A and 8B demonstrate the expression of HCVE1-HBsAg fusion protein in COS7 cells. Increasing amounts of sAg-encoding plasmid were expressed together with HCVE1-sAg fusion plasmid. The experiment is described in Example 3. The
15 capture antibody was MAb sAg, and the detecting antibody was MAb E2 (Fig. 8A) or MAb sAg (Fig. 8B).

Figures 9A and 9B show the sedimentation profile of virus-like particles expressed from COS7 cells transiently transfected with a 5:1 ratio of sAg:E1-sAg fusion protein. The experiment is described in Example 3. The capture antibody was MAb sAg,
20 and the detecting antibody was MAb E1 (Fig. 9A) or MAb sAg (Fig. 9B).

Figures 10A and 10B show the co-expression and secretion of E1-sAg and E2-sAg fusion proteins in COS7 cells. Increasing amounts of sAg-encoding plasmid were expressed together with constant amounts of both E1-sAg and E2-sAg fusion plasmids. The experiment is described in Example 4. In Fig. 10A, detecting antibodies for E1 and
25 E2 were employed, demonstrating the secretion into the media of both E1- and E2-derived polypeptides. In Fig. 10B, the detecting antibody was MAb sAg.

DETAILED DESCRIPTION OF THE INVENTION

A summary of standard techniques and procedures which may be employed in order to perform the invention follows. This summary is not a limitation on the invention but, rather, gives examples which may be used, but which are not required.

5 The practice of the present invention will employ, unless otherwise indicated, conventional techniques of molecular biology, microbiology, recombinant DNA, and immunology, which are within the skill of the art. Such techniques are explained fully in the literature e.g., Sambrook *Molecular Cloning; A Laboratory Manual, Second Edition* (1989); *DNA Cloning, Volumes I and ii* (D.N Glover ed. 1985); *Oligonucleotide*
10 *Synthesis* (M.J. Gait ed, 1984); *Animal Cell Culture* (R.I. Freshney ed. 1986); the *Methods in Enzymology* series (Academic Press, Inc.), especially volumes 154 & 155; *Gene Transfer Vectors for Mammalian Cells* (J.H. Miller and M.P. Calos eds. 1987, Cold Spring Harbor Laboratory); Mayer and Walker, eds. (1987), *Immunochemical Methods in Cell and Molecular Biology* (Academic Press, London).

15 All publications, patents and patent applications cited herein, whether *supra* or *infra*, are hereby incorporated by reference in their entireties.

 It must be noted that, as used in this specification and the appended claims, the singular forms "a", "an" and "the" include plural referents unless the content clearly dictates otherwise. Thus, for example, reference to "an antigen" includes a mixture of
20 two or more antigens, and the like.

 Standard abbreviations for nucleotides and amino acids are used in this specification. For example, the following amino acid abbreviations are used throughout the text:

25	Alanine: Ala (A)	Arginine: Arg (R)
	Asparagine: Asn (N)	Aspartic acid: Asp (D)
	Cysteine: Cys (C)	Glutamine: Gln (Q)
	Glutamic acid: Glu (E)	Glycine: Gly (G)
	Histidine: His (H)	Isoleucine: Ile (I)
	Leucine: Leu (L)	Lysine: Lys (K)
30	Methionine: Met (M)	Phenylalanine: Phe (F)

Proline: Pro (P)

Serine: Ser (S)

Threonine: Thr (T)

Tryptophan: Trp (W)

Tyrosine: Tyr (Y)

Valine: Val (V)

I. Definitions

5 In describing the present invention, the following terms will be employed, and are intended to be defined as indicated below.

The terms “polypeptide” and “protein” are used interchangeably herein and refer to a polymer of amino acid residues and are not limited to a minimum length of the product. Thus, peptides, oligopeptides, dimers, multimers, and the like, are included
10 within the definition. Both full-length proteins and fragments thereof are encompassed by the definition. The terms also include postexpression modifications of the polypeptide, for example, glycosylation, acetylation, phosphorylation and the like. Furthermore, for purposes of the present invention, a “polypeptide” refers to a protein which includes modifications, such as deletions, additions and substitutions (generally
15 conservative in nature), to the native sequence, so long as the protein maintains the desired activity. These modifications may be deliberate, as through site-directed mutagenesis, or may be accidental, such as through mutations of hosts which produce the proteins or errors due to PCR amplification.

An HCV polypeptide is a polypeptide, as defined above, derived from the HCV
20 polyprotein. HCV encodes a single polyprotein having more than 3000 amino acid residues (Choo et al. *Science* (1989) 244:359-362; Choo et al. *Proc. Natl. Acad. Sci. USA* (1991) 88:2451-2455; Han et al. *Proc. Natl. Acad. Sci. USA* (1991) 88:1711-1715). The polyprotein is processed co- and post-translationally into both structural and non-structural (NS) proteins.

25 In particular, several proteins are encoded by the HCV genome. The order and nomenclature of the cleavage products of the HCV polyprotein is as follows: NH₂-C-E1-E2-NS2-NS3-NS4a-NS4b-NS5a-NS5b-COOH. Initial cleavage of the polyprotein is catalyzed by host proteases which liberate three structural proteins, the N-terminal

nucleocapsid protein (termed "Core") and two envelope glycoproteins, "E1" (also known as E) and "E2" (also known as E2/NS1), as well as nonstructural (NS) proteins that contain the viral enzymes. The NS regions are termed NS2, NS3, NS4 and NS5. NS2 is an integral membrane protein with proteolytic activity. NS2, either alone or in combination with NS3, cleaves the NS2-NS3 sissle bond which in turn generates the NS3 N-terminus and releases a large polyprotein that includes both serine protease and RNA helicase activities. The NS3 protease serves to process the remaining polyprotein. Completion of polyprotein maturation is initiated by autocatalytic cleavage at the NS3-NS4a junction, catalyzed by the NS3 serine protease. Subsequent NS3-mediated cleavages of the HCV polyprotein appear to involve recognition of polyprotein cleavage junctions by an NS3 molecule of another polypeptide. In these reactions, NS3 liberates an NS3 cofactor (NS4a), two proteins with unknown function (NS4b and NS5a), and an RNA-dependent RNA polymerase (NS5b). Any one of these proteins, as well as immunogenic fragments thereof, will find use with the subject chimeric antigens.

The polypeptide for use in the chimeric antigens need not be physically derived from HCV, but may be synthetically or recombinantly produced. Moreover, the polypeptide may be derived from any of the various HCV strains, such as from strains 1, 2, 3 or 4 of HCV (described further below). A number of conserved and variable regions are known between these strains and, in general, the amino acid sequences of epitopes derived from these regions will have a high degree of sequence homology, e.g., amino acid sequence homology of more than 30%, preferably more than 40%, preferably more than 50% , preferably more than about 75%, more preferably more than about 80%-85%, preferably more than about 90%, and most preferably at least about 95%-98% sequence identity, or more, when the two sequences are aligned. Thus, for example, the term "E2" polypeptide refers to the native E2 protein from any of the various HCV strains, as well as E2 analogs, muteins and immunogenic fragments, as defined further below.

The terms "analog" and "mutein" refer to biologically active derivatives of the reference molecule, or fragments of such derivatives, that retain desired activity, such as immunological activity as described herein. In general, the term "analog" refers to compounds having a native polypeptide sequence and structure with one or more amino

acid additions, substitutions (generally conservative in nature) and/or deletions, relative to the native molecule, so long as the modifications do not destroy immunogenic activity. The term "mutein" refers to peptides having one or more peptide mimics ("peptoids"), such as those described in International Publ. No. WO 91/04282. Preferably, the analog
5 or mutein has at least the same immunoactivity as the native molecule. Methods for making polypeptide analogs and muteins are known in the art and are described further below.

Particularly preferred analogs include substitutions that are conservative in nature, i.e., those substitutions that take place within a family of amino acids that are related in
10 their side chains. Specifically, amino acids are generally divided into four families: (1) acidic -- aspartate and glutamate; (2) basic -- lysine, arginine, histidine; (3) non-polar -- alanine, valine, leucine, isoleucine, proline, phenylalanine, methionine, tryptophan; and (4) uncharged polar -- glycine, asparagine, glutamine, cysteine, serine, threonine, tyrosine. Phenylalanine, tryptophan, and tyrosine are sometimes classified as aromatic amino
15 acids. For example, it is reasonably predictable that an isolated replacement of leucine with isoleucine or valine, an aspartate with a glutamate, a threonine with a serine, or a similar conservative replacement of an amino acid with a structurally related amino acid, will not have a major effect on the biological activity. For example, the polypeptide of interest may include up to about 5-10 conservative or non-conservative amino acid
20 substitutions, or even up to about 15-25 conservative or non-conservative amino acid substitutions, or any integer between 5-25, so long as the desired function of the molecule remains intact. One of skill in the art may readily determine regions of the molecule of interest that can tolerate change by reference to Hopp/Woods and Kyte-Doolittle plots, well known in the art.

25 By "fragment" is intended a polypeptide consisting of only a part of the intact full-length polypeptide sequence and structure. The fragment can include a C-terminal deletion and/or an N-terminal deletion of the native polypeptide. An "immunogenic fragment" of a particular HCV protein will generally include at least about 5-10 contiguous amino acid residues of the full-length molecule, preferably at least about 15-
30 25 contiguous amino acid residues of the full-length molecule, and most preferably at

least about 20-50 or more contiguous amino acid residues of the full-length molecule, that define an epitope, or any integer between 5 amino acids and the full-length sequence, provided that the fragment in question retains the ability to elicit an immune response as defined below. For example, preferred immunogenic fragments, include but are not
5 limited to fragments of HCV Core that comprise, e.g., amino acids 10-45, 10-53, 67-88, 81-130, 86-100, 120-130, 121-135 and 121-170 of the polyprotein, numbered relative to the HCV-1a sequence presented in Choo et al. (1991) *Proc Natl Acad Sci USA* 88:2451, as well as defined epitopes derived from the c33c region of the HCV polyprotein, as well as any of the other various epitopes identified from the HCV core, E1, E2, NS3 and NS4
10 regions. See, e.g., Chien et al. *Proc. Natl. Acad. Sci. USA* (1992) 89:10011-10015; Chien et al. *J. Gastroent. Hepatol.* (1993) 8:S33-39; Chien et al. International Publ. No. WO 93/00365; Chien, D.Y. International Publ. No. WO 94/01778; and U.S. Patent No. 6,150,087, incorporated herein by reference in its entirety. Representative fragments of E1 and E2 polypeptides include C-terminally truncated variants of these molecules, such
15 as E1 polypeptides terminating at, e.g., amino acids 369 and lower, such as e.g., E1 polypeptides ending in amino acids 351, 352, 353 and so on, and E2 polypeptides, terminating at about amino acids 730, such as E2 polypeptides ending in for example amino acids 716, 717, 718 and so on. These molecules are described in, e.g., U.S. Patent No. 6,121,020, incorporated herein by reference in its entirety.

20 "Antigenic determinant" refers to the site on an antigen or hapten to which a specific antibody molecule or specific cell surface receptor binds.

The term "epitope" as used herein refers to a sequence of at least about 3 to 5, preferably about 5 to 10 or 15, and not more than about 1,000 amino acids (or any integer therebetween), which define a sequence that by itself or as part of a larger sequence, will
25 stimulate a host's immune system to make a cellular antigen-specific immune response when the antigen is presented, or a humoral antibody response. An epitope for use in the subject invention is not limited to a polypeptide having the exact sequence of the portion of the parent protein from which it is derived. Indeed, viral genomes are in a state of constant flux and contain several variable domains which exhibit relatively high degrees
30 of variability between isolates. Thus the term "epitope" encompasses sequences identical

to the native sequence, as well as modifications to the native sequence, such as deletions, additions and substitutions (generally conservative in nature).

Regions of a given polypeptide that include an epitope can be identified using any number of epitope mapping techniques, well known in the art. See, e.g., *Epitope Mapping Protocols* in *Methods in Molecular Biology*, Vol. 66 (Glenn E. Morris, Ed., 1996) Humana Press, Totowa, New Jersey. For example, linear epitopes may be determined by e.g., concurrently synthesizing large numbers of peptides on solid supports, the peptides corresponding to portions of the protein molecule, and reacting the peptides with antibodies while the peptides are still attached to the supports. Such techniques are known in the art and described in, e.g., U.S. Patent No. 4,708,871; Geysen et al. (1984) *Proc. Natl. Acad. Sci. USA* 81:3998-4002; Geysen et al. (1986) *Molec. Immunol.* 23:709-715, all incorporated herein by reference in their entireties. Similarly, conformational epitopes are readily identified by determining spatial conformation of amino acids such as by, e.g., x-ray crystallography and 2-dimensional nuclear magnetic resonance. See, e.g., *Epitope Mapping Protocols, supra*. Antigenic regions of proteins can also be identified using standard antigenicity and hydropathy plots, such as those calculated using, e.g., the Omega version 1.0 software program available from the Oxford Molecular Group. This computer program employs the Hopp/Woods method, Hopp et al., *Proc. Natl. Acad. Sci USA* (1981) 78:3824-3828 for determining antigenicity profiles, and the Kyte-Doolittle technique, Kyte et al., *J. Mol. Biol.* (1982) 157:105-132 for hydropathy plots.

As used herein, the term "conformational epitope" refers to a portion of a full-length protein, or an analog or mutein thereof, having structural features native to the amino acid sequence encoding the epitope within the full-length natural protein. Native structural features include, but are not limited to, glycosylation and three dimensional structure. Preferably, a conformational epitope is produced recombinantly and is expressed in a cell from which it is extractable under conditions which preserve its desired structural features, e.g. without denaturation of the epitope. Such cells include bacteria, yeast, insect, and mammalian cells. Expression and isolation of recombinant conformational epitopes from the HCV polyprotein are described in e.g., International

Publ. Nos. WO 96/04301, WO 94/01778, WO 95/33053, WO 92/08734, which applications are herein incorporated by reference in their entireties.

As used herein the term "T-cell epitope" refers to a feature of a peptide structure which is capable of inducing T-cell immunity towards the peptide structure or an associated hapten. T-cell epitopes generally comprise linear peptide determinants that assume extended conformations within the peptide-binding cleft of MHC molecules, (Unanue et al., *Science* (1987) 236:551-557). Conversion of polypeptides to MHC class II-associated linear peptide determinants (generally between 5 - 14 amino acids in length) is termed "antigen processing" which is carried out by antigen presenting cells (APCs). More particularly, a T-cell epitope is defined by local features of a short peptide structure, such as primary amino acid sequence properties involving charge and hydrophobicity, and certain types of secondary structure, such as helicity, that do not depend on the folding of the entire polypeptide. Further, it is believed that short peptides capable of recognition by helper T-cells are generally amphipathic structures comprising a hydrophobic side (for interaction with the MHC molecule) and a hydrophilic side (for interacting with the T-cell receptor), (Margalit et al., *Computer Prediction of T-cell Epitopes*, New Generation Vaccines Marcel-Dekker, Inc, ed. G.C. Woodrow et al., (1990) pp. 109-116) and further that the amphipathic structures have an α -helical configuration (see, e.g., Spouge et al. *J. Immunol.* (1987) 138:204-212; Berkower et al. *J. Immunol.* (1986) 136:2498-2503).

Hence, segments of proteins which include T-cell epitopes can be readily predicted using numerous computer programs. (See e.g., Margalit et al., *Computer Prediction of T-cell Epitopes*, New Generation Vaccines Marcel-Dekker, Inc, ed. G.C. Woodrow et al., (1990) pp. 109-116). Such programs generally compare the amino acid sequence of a peptide to sequences known to induce a T-cell response, and search for patterns of amino acids which are believed to be required for a T-cell epitope.

An "immunological response" to a polypeptide or composition is the development in a subject of a humoral and/or a cellular immune response to molecules present in the composition of interest. For purposes of the present invention, a "humoral immune response" refers to an immune response mediated by antibody molecules, while a

“cellular immune response” is one mediated by T-lymphocytes and/or other white blood cells. One important aspect of cellular immunity involves an antigen-specific response by cytolytic T-cells (“CTLs”). CTLs have specificity for peptide antigens that are presented in association with proteins encoded by the major histocompatibility complex (MHC) and expressed on the surfaces of cells. CTLs help induce and promote the intracellular destruction of intracellular microbes, or the lysis of cells infected with such microbes. Another aspect of cellular immunity involves an antigen-specific response by helper T-cells. Helper T-cells act to help stimulate the function, and focus the activity of, nonspecific effector cells against cells displaying peptide antigens in association with MHC molecules on their surface. A “cellular immune response” also refers to the production of cytokines, chemokines and other such molecules produced by activated T-cells and/or other white blood cells, including those derived from CD4+ and CD8+ T-cells.

A composition, such as an immunogenic composition, or vaccine that elicits a cellular immune response may serve to sensitize a vertebrate subject by the presentation of antigen in association with MHC molecules at the cell surface. The cell-mediated immune response is directed at, or near, cells presenting antigen at their surface. In addition, antigen-specific T-lymphocytes can be generated to allow for the future protection of an immunized host.

The ability of a particular antigen or composition to stimulate a cell-mediated immunological response may be determined by a number of assays, such as by lymphoproliferation (lymphocyte activation) assays, CTL cytotoxic cell assays, or by assaying for T-lymphocytes specific for the antigen in a sensitized subject. Such assays are well known in the art. See, e.g., Erickson et al., *J. Immunol.* (1993) 151:4189-4199; Doe et al., *Eur. J. Immunol.* (1994) 24:2369-2376; and the examples below.

Thus, an immunological response as used herein may be one which stimulates the production of CTLs, and/or the production or activation of helper T-cells. The antigen of interest may also elicit an antibody-mediated immune response. Hence, an immunological response may include one or more of the following effects: the production of antibodies by B-cells; and/or the activation of suppressor T-cells and/or $\gamma\delta$ T-cells

directed specifically to an antigen or antigens present in the composition or vaccine of interest. These responses may serve to neutralize infectivity, and/or mediate antibody-complement, or antibody dependent cell cytotoxicity (ADCC) to provide protection to an immunized host. Such responses can be determined using standard immunoassays and neutralization assays, well known in the art.

An "immunogenic" polypeptide or composition is one which elicits an immunological response as defined above.

A "recombinant" protein is a protein which retains the desired activity and which has been prepared by recombinant DNA techniques as described herein. In general, the gene of interest is cloned and then expressed in transformed organisms, as described further below. The host organism expresses the foreign gene to produce the protein under expression conditions.

By "isolated" is meant, when referring to a polypeptide, that the indicated molecule is separate and discrete from the whole organism with which the molecule is found in nature or is present in the substantial absence of other biological macromolecules of the same type. The term "isolated" with respect to a polynucleotide is a nucleic acid molecule devoid, in whole or part, of sequences normally associated with it in nature; or a sequence, as it exists in nature, but having heterologous sequences in association therewith; or a molecule disassociated from the chromosome.

"Homology" refers to the percent identity between two polynucleotide or two polypeptide moieties. Two DNA, or two polypeptide sequences are "substantially homologous" to each other when the sequences exhibit at least about 50% , preferably at least about 75%, more preferably at least about 80%-85%, preferably at least about 90%, and most preferably at least about 95%-98% sequence identity over a defined length of the molecules. As used herein, substantially homologous also refers to sequences showing complete identity to the specified DNA or polypeptide sequence.

In general, "identity" refers to an exact nucleotide-to-nucleotide or amino acid-to-amino acid correspondence of two polynucleotides or polypeptide sequences, respectively. Percent identity can be determined by a direct comparison of the sequence information between two molecules by aligning the sequences, counting the exact number

of matches between the two aligned sequences, dividing by the length of the shorter sequence, and multiplying the result by 100. Readily available computer programs can be used to aid in the analysis, such as ALIGN, Dayhoff, M.O. in *Atlas of Protein Sequence and Structure* M.O. Dayhoff ed., 5 Suppl. 3:353-358, National biomedical Research Foundation, Washington, DC, which adapts the local homology algorithm of Smith and Waterman *Advances in Appl. Math.* 2:482-489, 1981 for peptide analysis. Programs for determining nucleotide sequence identity are available in the Wisconsin Sequence Analysis Package, Version 8 (available from Genetics Computer Group, Madison, WI) for example, the BESTFIT, FASTA and GAP programs, which also rely on the Smith and Waterman algorithm. These programs are readily utilized with the default parameters recommended by the manufacturer and described in the Wisconsin Sequence Analysis Package referred to above. For example, percent identity of a particular nucleotide sequence to a reference sequence can be determined using the homology algorithm of Smith and Waterman with a default scoring table and a gap penalty of six nucleotide positions.

Another method of establishing percent identity in the context of the present invention is to use the MPSRCH package of programs copyrighted by the University of Edinburgh, developed by John F. Collins and Shane S. Sturrok, and distributed by IntelliGenetics, Inc. (Mountain View, CA). From this suite of packages the Smith-Waterman algorithm can be employed where default parameters are used for the scoring table (for example, gap open penalty of 12, gap extension penalty of one, and a gap of six). From the data generated the "Match" value reflects "sequence identity." Other suitable programs for calculating the percent identity or similarity between sequences are generally known in the art, for example, another alignment program is BLAST, used with default parameters. For example, BLASTN and BLASTP can be used using the following default parameters: genetic code = standard; filter = none; strand = both; cutoff = 60; expect = 10; Matrix = BLOSUM62; Descriptions = 50 sequences; sort by = HIGH SCORE; Databases = non-redundant, GenBank + EMBL + DDBJ + PDB + GenBank CDS translations + Swiss protein + Spupdate + PIR. Details of these programs can be found at the following internet address: <http://www.ncbi.nlm.gov/cgi-bin/BLAST>.

Alternatively, homology can be determined by hybridization of polynucleotides under conditions which form stable duplexes between homologous regions, followed by digestion with single-stranded-specific nuclease(s), and size determination of the digested fragments. DNA sequences that are substantially homologous can be identified in a
5 Southern hybridization experiment under, for example, stringent conditions, as defined for that particular system. Defining appropriate hybridization conditions is within the skill of the art. See, e.g., Sambrook et al., *supra*; *DNA Cloning, supra*; *Nucleic Acid Hybridization, supra*.

A "coding sequence" or a sequence which "encodes" a selected polypeptide, is a
10 nucleic acid molecule which is transcribed (in the case of DNA) and translated (in the case of mRNA) into a polypeptide *in vitro* or *in vivo* when placed under the control of appropriate regulatory sequences. The boundaries of the coding sequence are determined by a start codon at the 5' (amino) terminus and a translation stop codon at the 3' (carboxy) terminus. A transcription termination sequence may be located 3' to the coding sequence.

15 "Operably linked" refers to an arrangement of elements wherein the components so described are configured so as to perform their desired function. Thus, a given promoter operably linked to a coding sequence is capable of effecting the expression of the coding sequence when the proper transcription factors, etc., are present. The promoter need not be contiguous with the coding sequence, so long as it functions to
20 direct the expression thereof. Thus, for example, intervening untranslated yet transcribed sequences can be present between the promoter sequence and the coding sequence, as can transcribed introns, and the promoter sequence can still be considered "operably linked" to the coding sequence.

"Recombinant" as used herein to describe a nucleic acid molecule means a
25 polynucleotide of genomic, cDNA, viral, semisynthetic, or synthetic origin which, by virtue of its origin or manipulation is not associated with all or a portion of the polynucleotide with which it is associated in nature. The term "recombinant" as used with respect to a protein or polypeptide means a polypeptide produced by expression of a recombinant polynucleotide. In general, the gene of interest is cloned and then expressed

in transformed organisms, as described further below. The host organism expresses the foreign gene to produce the protein under expression conditions.

5 A “control element” refers to a polynucleotide sequence which aids in the expression of a coding sequence to which it is linked. The term includes promoters, transcription termination sequences, upstream regulatory domains, polyadenylation signals, untranslated regions, including 5'-UTRs and 3'-UTRs and when appropriate, leader sequences and enhancers, which collectively provide for the transcription and translation of a coding sequence in a host cell.

10 A “promoter” as used herein is a DNA regulatory region capable of binding RNA polymerase in a host cell and initiating transcription of a downstream (3' direction) coding sequence operably linked thereto. For purposes of the present invention, a promoter sequence includes the minimum number of bases or elements necessary to initiate transcription of a gene of interest at levels detectable above background. Within the promoter sequence is a transcription initiation site, as well as protein binding domains
15 (consensus sequences) responsible for the binding of RNA polymerase. Eucaryotic promoters will often, but not always, contain “TATA” boxes and “CAT” boxes.

A control sequence “directs the transcription” of a coding sequence in a cell when RNA polymerase will bind the promoter sequence and transcribe the coding sequence into mRNA, which is then translated into the polypeptide encoded by the coding
20 sequence.

“Expression cassette” or “expression construct” refers to an assembly which is capable of directing the expression of the sequence(s) or gene(s) of interest. The expression cassette includes control elements, as described above, such as a promoter which is operably linked to (so as to direct transcription of) the sequence(s) or gene(s) of
25 interest, and often includes a polyadenylation sequence as well. Within certain embodiments of the invention, the expression cassette described herein may be contained within a plasmid construct. In addition to the components of the expression cassette, the plasmid construct may also include, one or more selectable markers, a signal which allows the plasmid construct to exist as single-stranded DNA (e.g., a M13 origin of

replication), at least one multiple cloning site, and a “mammalian” origin of replication (e.g., a SV40 or adenovirus origin of replication).

“Transformation,” as used herein, refers to the insertion of an exogenous polynucleotide into a host cell, irrespective of the method used for insertion: for example, transformation by direct uptake, transfection, infection, and the like. For particular methods of transfection, see further below. The exogenous polynucleotide may be maintained as a nonintegrated vector, for example, an episome, or alternatively, may be integrated into the host genome.

A “host cell” is a cell which has been transformed, or is capable of transformation, by an exogenous DNA sequence.

By “nucleic acid immunization” is meant the introduction of a nucleic acid molecule encoding one or more selected antigens into a host cell, for the *in vivo* expression of the antigen or antigens. The nucleic acid molecule can be introduced directly into the recipient subject, such as by injection, inhalation, oral, intranasal and mucosal administration, or the like, or can be introduced *ex vivo*, into cells which have been removed from the host. In the latter case, the transformed cells are reintroduced into the subject where an immune response can be mounted against the antigen encoded by the nucleic acid molecule.

As used herein, “treatment” refers to any of (i) the prevention of infection or reinfection, as in a traditional vaccine, (ii) the reduction or elimination of symptoms, and (iii) the substantial or complete elimination of the pathogen in question. Treatment may be effected prophylactically (prior to infection) or therapeutically (following infection).

By “vertebrate subject” is meant any member of the subphylum cordata, including, without limitation, humans and other primates, including non-human primates such as chimpanzees and other apes and monkey species; farm animals such as cattle, sheep, pigs, goats and horses; domestic mammals such as dogs and cats; laboratory animals including rodents such as mice, rats and guinea pigs; birds, including domestic, wild and game birds such as chickens, turkeys and other gallinaceous birds, ducks, geese, and the like. The term does not denote a particular age. Thus, both adult and newborn individuals are intended to be covered. The invention described herein is intended for use

in any of the above vertebrate species, since the immune systems of all of these vertebrates operate similarly.

II. Modes of Carrying out the Invention

Before describing the present invention in detail, it is to be understood that this invention is not limited to particular formulations or process parameters as such may, of course, vary. It is also to be understood that the terminology used herein is for the purpose of describing particular embodiments of the invention only, and is not intended to be limiting.

Although a number of compositions and methods similar or equivalent to those described herein can be used in the practice of the present invention, the preferred materials and methods are described herein.

As noted above, chimeric antigens which combine HBsAg with portions of HCV proteins enhance the presentation to the immune system of weakly immunogenic HCV proteins due to the strong antigenicity of HBsAg. Virus-like particles typically contain a membrane envelope in which are embedded one or more viral envelope proteins. Virus-like particles are secreted by a cell infected with a virus or a cell transfected with a nucleic acid molecule encoding one or more viral proteins.

Virus-like particles are an especially advantageous form of antigen presentation. Virus-like particles containing HBsAg chimeras combine the highly antigenic nature of HBsAg itself with the desirable presentation of other antigens at the surface of a particulate preparation.

The present invention provides methods and materials which enable the production of chimeric HBV/HCV antigens in the form of virus-like particles for the production of immunogenic compositions, such as vaccines. Utilizing co-expression of HBsAg together with the chimeric antigens, it is now possible to include large segments of HCV envelope glycoproteins fused to HBsAg in the form of virus-like particles. Such particles are especially well suited for use in immunogenic compositions.

The strategy for preparing HBV/HCV virus-like particles depends first on the production of one or more chimeric antigens for display in the particles. Such chimeric

antigens are fusion proteins that comprise a substantially complete S domain of an HBsAg polypeptide (termed "sAg" herein) and an immunogenic HCV polypeptide or fragment thereof. An S domain of HBsAg, or any other polypeptide of the invention, is "substantially complete" if it contains the native sequence of the polypeptide with or without minor deletions of one or a few amino acids from either the N-terminal or C-terminal regions or within the polypeptide. For example the HBsAg S domain can be truncated by a few amino acids, *i.e.*, up to about 3, 5, 7, or 10 amino acids, without greatly affecting either its antigenicity or ability to cause the formation of virus-like particles. Linker sequences of any desired length, preferably no more than a few amino acids, can be added between the polypeptides joined together to form the fusion protein. Either preS2 (formerly called preS) or both preS2 and preS1 domains of HBsAg can also be included at the amino terminus of the HBsAg polypeptide if desired.

Valenzuela, et al. (1982) *Nature* 298:347-350, describes the gene for HbsAg. See, also, Valenzuela, et al. (1979) *Nature* 280:815-819. Proteins derived from the HBV surface, such as the surface antigen, sAg, as well as the presurface sequences, preS1 and preS2, and any combination of these sequences, can spontaneously form particles upon expression in a suitable host cell, such as upon expression in mammalian, insect, yeast or *Xenopus* cells. Thus, as explained above, HCV/HBV virus-like particles for use in the present invention can include particle-forming polypeptides of sAg, preS1 and/or preS2, as well as particle-forming polypeptides from any combination of the above, such as sAg/preS1, sAg/preS2, and sAg/preS1/preS2. See, e.g., "HBV Vaccines - from the laboratory to license: a case study" in Mackett, M. and Williamson, J.D., *Human Vaccines and Vaccination*, pp. 159-176, for a discussion of HBV structure; and U.S. Patent Nos. 4,722,840, 5,098,704, 5,324,513, 5,965,140, incorporated herein by reference in their entireties, Beames et al., *J. Virol.* (1995) 69:6833-6838, Birnbaum et al., *J. Virol.* (1990) 64:3319-3330, Zhou et al., *J. Virol.* (1991) 65:5457-5464, for descriptions of the recombinant production of various HBV particles.

HCV has a genome that contains a single open reading frame of approximately 9.5 kb, which is transcribed into a polyprotein. The HCV polyprotein is cleaved to form at least ten distinct products, which are NH₂--Core--E1--E2--p7--NS2--NS3--NS4a--

NS4b--NS5a--NS5b--COOH. The HCV E1 and E2 proteins are postranslationally glycosylated. The full-length sequence of the polyprotein is disclosed in European Publ. No. 388,232 and U.S. Patent No. 6,150,087, incorporated herein by reference in their entireties. Moreover, sequences for the above HCV polyprotein products, and
5 immunogenic polypeptides derived therefrom, are known (see, e.g., U.S. Patent No. 5,350,671, incorporated herein by reference in its entirety). For example, a number of general and specific immunogenic polypeptides, derived from the HCV polyprotein, have been described. See, e.g., Houghton et al., European Publ. Nos. 318,216 and 388,232; Choo et al. *Science* (1989) 244:359-362; Kuo et al. *Science* (1989) 244:362-364;
10 Houghton et al. *Hepatology* (1991) 14:381-388; Chien et al. *Proc. Natl. Acad. Sci. USA* (1992) 89:10011-10015; Chien et al. *J. Gastroent. Hepatol.* (1993) 8:S33-39; Chien et al., International Publ. No. WO 93/00365; Chien, D.Y., International Publ. No. WO 94/01778. These publications provide an extensive background on HCV generally, as well as on the manufacture and uses of HCV polypeptide immunological reagents. For
15 brevity, therefore, the disclosure of these publications is incorporated herein by reference.

Any desired HCV polypeptide can be utilized as part of the chimeric antigen, including, for example, the E1 and/or E2 envelope glycoproteins of HCV. The E1 glycoprotein corresponds to amino acid residues 192 to 383, and E2 extends from approximately amino acid 384 to amino acid 746 in most HCV strains. See Choo et al.
20 (1991) *Proc. Natl. Acad. Sci. USA* 88:2451-2455. Preferred E1 polypeptides are those beginning at any amino acid residue from any of positions 192-360 of the HCV polyprotein and extending any desired length up to and including residue 383. Preferred E2 polypeptides are those beginning at any amino acid residue from positions 384-725 of the HCV polyprotein and extending any desired length up to and including residue 746.
25 A fragment of the E1 or E2 glycoproteins which is utilized in the chimeric antigen should preferably comprise an epitope, domain, or other structural unit which is immunogenic.

An E1 or E2 glycoprotein or fragment thereof for use in a chimeric antigen, will preferably retain or resemble its native conformation. If a substantially native conformation is retained for the HCV polypeptide in the fusion protein containing

HBsAg, then antibodies generated to the HCV polypeptide will recognize and bind to the corresponding polypeptide in HCV.

Other HCV polypeptides may also be used in the chimeric antigens of the invention. For example, HCV polypeptides derived from the Core region, such as
5 polypeptides derived from the region found between amino acids 1-191; amino acids 10-53; amino acids 10-45; amino acids 67-88; amino acids 86-100; 81-130; amino acids 121-135; amino acids 120-130; amino acids 121-170; and any of the Core epitopes identified in, e.g., Houghton et al., U.S. Patent No. 5,350,671; Chien et al. *Proc. Natl. Acad. Sci. USA* (1992) 89:10011-10015; Chien et al. *J. Gastroent. Hepatol.* (1993) 8:S33-39; Chien
10 et al., International Publ. No. WO 93/00365; Chien, D.Y., International Publ. No. WO 94/01778; and U.S. Patent No. 6,150,087, the disclosures of which are incorporated herein by reference in their entireties, will find use with the subject chimeric molecules.

Additionally, polypeptides derived from the nonstructural regions of the virus will also find use herein. The NS3/4a region of the HCV polyprotein has been described and
15 the amino acid sequence and overall structure of the protein are disclosed in Yao et al. *Structure* (November 1999) 7:1353-1363. See, also, Dasmahapatra et al., U.S. Patent No. 5,843,752, incorporated herein by reference in its entirety. As explained above, either the native sequence or immunogenic analogs can be used in the subject formulations. Dasmahapatra et al., U.S. Patent No. 5,843,752 and Zhang et al., U.S. Patent No.
20 5,990,276, both describe analogs of NS3/4a and methods of making the same.

Additionally, multiple epitope fusion antigens (termed "MEFAs"), as described in International Publ. No. WO 97/44469, may be used in the subject chimeras. Such MEFAs include multiple epitopes derived from two or more of the various viral regions. The epitopes are preferably from more than one HCV strain, thus providing the added
25 ability to protect against multiple strains of HCV in a single vaccine.

Moreover, polypeptides for use in the subject chimeras may be derived from the NS3 region of the HCV polyprotein. A number of such polypeptides are known, including, but not limited to polypeptides derived from the c33c and c100 regions, as well as fusion proteins comprising an NS3 epitope, such as c25. These and other NS3
30 polypeptides are useful in the present compositions and are known in the art and

described in, e.g., Houghton et al, U.S. Patent No. 5,350,671; Chien et al. *Proc. Natl. Acad. Sci. USA* (1992) 89:10011-10015; Chien et al. *J. Gastroent. Hepatol.* (1993) 8:S33-39; Chien et al., International Publ. No. WO 93/00365; Chien, D.Y., International Publ. No. WO 94/01778; and U.S. Patent No. 6,150,087, the disclosures of which are
5 incorporated herein by reference in their entireties.

It is readily apparent that a multitude of HCV polypeptides may be used in the subject chimeric molecules or may be coadministered therewith, in order to provide an immune response against the HCV antigen in question.

As explained above, the HCV antigens may be used in their entireties or
10 immunogenic fragments thereof, as well as immunogenic variants, can be combined with an HBsAg polypeptide to form the chimeric antigen by fusion of the gene sequences encoding the desired polypeptide sequences, in proper reading frame, using standard techniques available in the art. Thus, the HBV or HCV polypeptides described for use in the invention can be modified by deletions, insertions, or conservative amino acid
15 substitutions, provided that a substantially native conformation is retained for the HCV epitope intended for use as an immunogen. Preferably, the HCV polypeptide or polypeptide fragment is chosen so that, when expressed in a fusion protein with HBsAg, it substantially retains the native conformation of the corresponding portion of the HCV protein from which it was derived, *i.e.*, the approximate conformation existing in a
20 mature HCV virion.

It should be noted that for convenience, the various HCV regions are generally defined with respect to the amino acid number relative to the polyprotein encoded by the genome of HCV-1a, as described in Choo et al. (1991) *Proc Natl Acad Sci USA* 88:2451, with the initiator methionine being designated position 1. However, the polypeptides for
25 use with the present invention are not limited to those derived from the HCV-1a sequence. Any strain or isolate of HCV can serve as the basis for providing antigenic sequences for use with the invention. In this regard, the corresponding regions in another HCV isolate can be readily determined by aligning sequences from the two isolates in a manner that brings the sequences into maximum alignment.

Various strains and isolates of HCV are known in the art, which differ from one another by changes in nucleotide and amino acid sequence. For example, isolate HCV J1.1 is described in Kubo *et al.* (1989) Japan. Nucl. Acids Res. 17:10367-10372; Takeuchi *et al.* (1990) Gene 91:287-291; Takeuchi *et al.* (1990) J. Gen. Virol. 71:3027-3033; and Takeuchi *et al.* (1990) Nucl. Acids Res. 18:4626. The complete coding sequences of two independent isolates, HCV-J and BK, are described by Kato *et al.*, (1990) Proc. Natl. Acad. Sci. USA 87:9524-9528 and Takamizawa *et al.*, (1991) J. Virol. 65:1105-1113, respectively. HCV-1 isolates are described by Choo *et al.* (1990) Brit. Med. Bull. 46:423-441; Choo *et al.* (1991) Proc. Natl. Acad. Sci. USA 88:2451-2455 and Han *et al.* (1991) Proc. Natl. Acad. Sci. USA 88:1711-1715. HCV isolates HC-J1 and HC-J4 are described in Okamoto *et al.* (1991) Japan J. Exp. Med. 60:167-177. HCV isolates HCT 18~, HCT 23, Th, HCT 27, EC1 and EC10 are described in Weiner *et al.* (1991) Virol. 180:842-848. HCV isolates Pt-1, HCV-K1 and HCV-K2 are described in Enomoto *et al.* (1990) Biochem. Biophys. Res. Commun. 170:1021-1025. HCV isolates A, C, D & E are described in Tsukiyama-Kohara *et al.* (1991) Virus Genes 5:243-254.

Coding sequences for naturally occurring HCV polypeptides for use in the nucleic acid molecules or vectors of the invention can be obtained from any of the above cited strains of HCV or from newly discovered isolates isolated from tissues or fluids of infected patients. In choosing HCV polypeptides or their fragments from different HCV strains for use in a chimeric antigen of the invention, it is preferred that the nucleotide or amino acid sequences be aligned for maximum overlap between strains. This can be accomplished, for example, by compensating for insertions or deletions of amino acids so that the greatest possible overlap between strains is obtained prior to selecting a sequence for incorporation in the chimeric antigen. If a sequence disclosed herein is selected from another HCV strain, then preferably the corresponding sequence, *i.e.*, the sequence which is aligned to be identical at the greatest possible number of residues to the disclosed sequence, will be selected. Modified sequences which do not occur in nature can also be used. A "nucleic acid molecule" according to the invention can be any nucleic acid such as DNA or RNA, either single or double stranded, or any analog or chemical derivative

thereof which encodes a fusion protein of the invention or portion thereof or any HBsAg or portion thereof.

Nucleic acid molecules of the invention can be cloned into expression vectors and transformed into, for example, bacterial, yeast, plant, insect, or mammalian cells so that the chimeric antigens and virus-like particles of the invention can be expressed in and isolated from cell culture. Nucleic acid molecules can be contained within a plasmid, such as pBR322, pUC, ColE1, or related plasmids such as pCMV6a (see U.S. Patent 5,688,688) or plasmids derived therefrom. Nucleic acid molecules can also be contained within a viral vector, such as any vector derived from adenovirus, Sindbis virus, simian virus 40, cytomegalovirus, and retroviruses such as murine sarcoma virus, mouse mammary tumor virus, Moloney murine leukemia virus, and Rous sarcoma virus. Bacterial vectors, such as *Salmonella* spp., *Yersinia enterocolitica*, *Shigella* spp., *Vibrio cholerae*, *Mycobacterium* strain BCG, and *Listeria monocytogenes* can also be used. Minichromosomes such as MC and MC1, bacteriophages, cosmids, and replicons can be used as well.

Any suitable expression vector can be constructed or utilized to express any form of HBsAg or any chimeric antigen of the invention. A preferred vector is pCMVII, a pUC19-based cloning vector designed for expression in mammalian cells. pCMVII comprises the following elements: human CMV IE enhancer/promoter, human CMV intron A, a human tissue plasminogen activator (tPA) leader, a bovine growth hormone poly A terminator (BGHt), a ColE1 origin of replication, and an *Amp^R* ampicillin resistance gene (Figs. 1A and 1B-1F, SEQ ID NO:1). pCMVII-pS2-sAg can be used for expression of preS2-HBsAg (Figs. 2A and 2B-2I, SEQ ID NOS:2 and 3). In pCMVII-pS2-sAg, the coding sequences for the preS2 and S domains of HBsAg have been inserted into pCMVII between CMV intron A and BGHt; this vector can also be modified by adding the coding sequences for the preS1 domain or removing the preS2 domain. Chimeric antigens containing HBsAg together with epitopes of HCV proteins can be constructed similar to pCMVII opti 330 E1/sAg (Figs. 3A and 3B-3I, SEQ ID NOS:4 and 5) or pCMVII-E2661-sAg (Figs. 4A and 4B-4F, SEQ ID NOS:6 and 7). These vectors are provided by way of example and are not intended to limit the scope of the invention.

Isolated and purified pCMVII vectors containing an insert encoding HBsAg or a chimeric antigen can be dissolved in sterile 0.9% saline buffer or another suitable buffer prior to use.

5 The expression of chimeric antigens for use as immunogens requires transfection of eukaryotic cells with an expression vector. Any suitable eukaryotic cell line can be used, for example CHO cells or COS cells. A nucleic acid molecule that encodes a chimeric antigen can be incorporated into any vector suitable for transfection, for example a plasmid vector or a viral vector. If a viral vector is used, it will preferably produce defective, non-infectious viral particles so that the risk of contamination with
10 infectious virus in a vaccine preparation is minimized.

Transfection can be performed by any known method and can result in either transient transfection or stable transfection. Stable transfection is preferred to establish a cell line producing HBV/HCV virus-like particles. Methods for obtaining stable transfection are well known and include, for example, selection for spontaneously stable
15 transfectants, transfection with immortalizing genes (*see, e.g.*, Katakura et al., *Methods Cell Biol.* (1998) 57:69-91), and selection for genes providing resistance to antibiotics such as hygromycin B and neomycin. A preferred method for CHO cells involves selection for cells transfected with dihydrofolate reductase followed by amplification of the transgene using methotrexate (*see* Wurm et al., *Ann. N.Y. Acad. Sci.* (1996) 782:70-
20 78).

Co-expression of chimeric antigens with HBsAg results in formation and secretion of virus-like particles. Optimum secretion of virus-like particles requires a sufficient quantity of expression of HBsAg in relation to the expression of chimeric antigen, and higher quantities of HBsAg expression generally improve the efficiency of
25 chimeric antigen secretion. Secretion of chimeric antigen as virus-like particles can be optimized by varying the ratio of the coding sequences for HBsAg to the coding sequences for the chimeric antigen. Generally, useful secretion of virus-like particles will be obtained at a ratio of HBsAg coding sequences to chimeric antigen coding sequences of about 1:1, 2:1, 3:1, 4:1, 5:1, 7:1, 10:1, 20:1, 30:1, 40:1, 50:1, or 100:1. Ratios less
30 than 1:1 provide reduced yield of virus-like particles containing chimeric antigen, while

high ratios greater than 100:1 dilute out the chimeric antigen and eventually will limit the utility of the particles because of dilution of the chimeric antigen with HBsAg.

One strategy for transfecting host cells with the vectors of the invention, either for *in vitro* production of virus-like particles or for immunization of a host organism using a nucleic acid vaccine, is to provide two or more separate vectors, one that encodes HBsAg and one or more that each encode a chimeric antigen. Another strategy is to include both HBsAg and one or more chimeric antigens in a single construct. For example, an expression vector having a single open reading frame could have a coding sequence for HBsAg under control of a promoter, followed by sequences encoding one or more chimeric antigens, each preceded by an internal ribosomal entry site (IRES, *see, e.g.*, Martinez-Salas (1999) Curr. Op. Biotechnol. 10: 458-464). Thus, the invention encompasses the use either of a single immunogenic polypeptide or multiple distinct immunogenic polypeptides in a single virus-like particle. Optionally, expression of immunogenic polypeptides can be regulated by including promoter and/or enhancer sequences in the expression vector which respond to activators introduced into the transfected cells.

Cells that have been transfected with plasmid or viral vectors expressing chimeric HBV/HCV antigens together with HBsAg can be analyzed to determine whether each antigen is being expressed and secreted in the form of virus-like particles. Any standard immunological assay can be used to detect HBV and HCV antigens, including, for example, chemiluminescence assays (*see, e.g.*, Magic Lite assay in Examples 1-4; Woodhead, J.S. and Weeks, I. (1989) J. Biolumin. Chemilumin. 4:611-14), ELISA, and radioimmunoassays. Where an antigen is produced by transfected cells in culture, the amount of secretion of the antigen into the culture medium can be ascertained by comparing the amount of antigen in the culture medium with the amount remaining in lysed cells. The presence of virus-like particles can be confirmed by sedimentation of such particles from the culture medium into a density gradient, for example a sucrose density gradient (*see* Examples 1-3).

While expression in a mammalian cell line is preferred, other systems may also be employed to express virus-like particles of the invention. For example, yeast cell culture

may be used (*see* U.S. Patent 5,098,704, incorporated herein by reference), in which case virus-like particles can be harvested by disrupting the cells using agitation with glass beads or another suitable method.

5 Generally, the proteins of the subject invention will naturally aggregate to form particles in the expression host. The particles may be enveloped having a lipid membrane coat, which may or may not include membrane proteins encoded by the virus. Alternatively, the particles may not include the lipid membrane or the membrane may be present initially or may be removed, in whole or in part. U.S. Patent Nos. 4,722,840 and 5,965,140, incorporated herein by reference in their entireties, describe hybrid particles
10 comprised of a particle-forming fragment of a structural protein from a virus, such as a particle-forming fragment of hepatitis B virus (HBV) surface antigen (HBsAg), fused to a heterologous polypeptide.

HBV/HCV virus-like particles can be purified after being harvested from a culture medium or cell suspension and before being used in an immunogenic composition. Any
15 method can be used that is known to separate virus-like particles or viruses from surrounding proteins, lipids, nucleic acids, membranes, intact cells, and the like. Especially preferred are affinity chromatography methods; for example, an immobilized monoclonal antibody specific for HBsAg can be used. Additional suitable methods are gel filtration chromatography, ion exchange chromatography, and density gradient
20 sedimentation. Methods for isolated chimeric virus-like particles are described in, e.g., U.S. Patent Nos. 4,722,840 and 5,965,140, incorporated herein by reference in their entireties.

Any composition of the invention, such as a fusion protein, a virus-like particle, or a nucleic acid molecule, can be used as an "immunogenic composition." An
25 immunogenic composition preferably generates an immune response, as defined above, such as an antibody or a T-cell response, in a mammal to whom it is administered. An immunogenic composition of the invention can be, but is not limited to, a vaccine or a combination vaccine, e.g., an immunogenic composition that produces an immune response to more than one immunogen. Moreover, the chimeric proteins of the invention

may be formulated into "antigenic compositions," e.g., compositions that include epitopes to which a specific antibody molecule or specific cell surface receptor binds.

Immunogenic and antigenic compositions containing HBV/HCV virus-like particles or a nucleic acid molecule encoding proteins which form virus-like particles can be administered to a mammal, such as a mouse, rabbit, baboon, chimpanzee, or human, to elicit anti-HCV antibodies *in vivo*. Injection of an immunogenic composition of the invention preferably results in the synthesis of virus-like particles in the host. Therefore, Compositions comprising nucleic acids should include sequences encoding both HBsAg and chimeric antigens such as HBsAg-E1 and/or HBsAg-E2 fusion proteins, as well as fusions with other HCV polypeptides. The weight:weight ratio of nucleic acids encoding HBsAg to nucleic acids encoding chimeric antigens is preferably 1:1, 2:1, 5:1, 10:1, 20:1, 30:1, 50:1, or 100:1 and results in the formation of virus-like particles in the cells of the host and their secretion within the host. More preferably, the ratio is in the range of 5:1 to 20:1.

Virus-like particles or nucleic acid molecules of an antigenic or immunogenic composition can be combined with adjuvants, immunostimulatory molecules, or carriers, including but not limited to, MF59 (described below), poly(dl-lactide-co-glycolide) microparticles (PLG, *see, e.g.*, Delgado et al. (1999) Vaccine 17: 2927-2938), LT toxins, immune stimulating complexes (ISCOMS, *see, e.g.*, Mowat et al. (1999) Immunol. Lett. 65: 133-140), and QS21 (*see* Singh & O'Hagan (1999) Nat. Biotechnol. 17: 1075-1081).

For example, preferred adjuvants to enhance effectiveness of the composition include, but are not limited to: (1) aluminum salts (alum), such as aluminum hydroxide, aluminum phosphate, aluminum sulfate, etc; (2) oil-in-water emulsion formulations (with or without other specific immunostimulating agents such as muramyl peptides (*see* below) or bacterial cell wall components), such as for example (a) MF59 (PCT Publ. No. WO 90/14837), containing 5% Squalene, 0.5% Tween 80, and 0.5% Span 85 (optionally containing various amounts of MTP-PE (*see* below), although not required) formulated into submicron particles using a microfluidizer such as Model 110Y microfluidizer (Microfluidics, Newton, MA), (b) SAF, containing 10% Squalene, 0.4% Tween 80, 5% pluronic-blocked polymer L121, and thr-MDP (*see* below) either microfluidized into a

submicron emulsion or vortexed to generate a larger particle size emulsion, and (c) RibiTM adjuvant system (RAS), (Ribi Immunochem, Hamilton, MT) containing 2% Squalene, 0.2% Tween 80, and one or more bacterial cell wall components from the group consisting of monophosphorylipid A (MPL), trehalose dimycolate (TDM), and cell wall skeleton (CWS), preferably MPL + CWS (DetoxTM); (3) saponin adjuvants, such as StimulonTM (Cambridge Bioscience, Worcester, MA) may be used or particles generated therefrom such as ISCOMs (immunostimulating complexes); (4) Complete Freund's Adjuvant (CFA) and Incomplete Freund's Adjuvant (IFA); (5) cytokines, such as interleukins (e.g., IL-1, IL-2, IL-4, IL-5, IL-6, IL-7, IL-12, *etc.*), interferons (e.g., gamma interferon), macrophage colony stimulating factor (M-CSF), tumor necrosis factor (TNF), etc; (6) detoxified mutants of a bacterial ADP-ribosylating toxin such as a cholera toxin (CT), a pertussis toxin (PT), or an E. coli heat-labile toxin (LT), particularly LT-K63, LT-R72, CT-S109, PT-K9/G129; see, e.g., WO 93/13302 and WO 92/19265; (7) other substances that act as immunostimulating agents to enhance the effectiveness of the composition; and (8) microparticles with adsorbed macromolecules, as described in copending U.S. Patent Application Serial No. 09/285,855 (filed April 2, 1999) and international Patent Application Serial No. PCT/US99/17308 (filed July 29, 1999). Alum and MF59 are preferred.

As mentioned above, muramyl peptides include, but are not limited to, N-acetyl-muramyl-L-threonyl-D-isoglutamine (thr-MDP), N-acetyl-normuramyl-L-alanyl-D-isoglutamine (nor-MDP), N-acetylmuramyl-L-alanyl-D-isoglutaminyl-L-alanine-2-(1'-2'-dipalmitoyl-*sn*-glycero-3-hydroxyphosphoryloxy)-ethylamine (MTP-PE), *etc.*

An immunogenic composition may also comprise a pharmaceutically acceptable carrier. Pharmaceutically acceptable carriers are well known to those in the art. Such carriers include, but are not limited to, large, slowly metabolized macromolecules, such as proteins, polysaccharides, polylactic acids, polyglycolic acids, polymeric amino acids, amino acid copolymers, and inactive virus particles. Liposomes, such as those described in U.S. 5,422,120, WO 95/13796, WO 91/14445, or EP 524,968 B1, as well as poly(dl-lactide-co-glycolide) microparticles (PLG, *see, e.g.,* Delgado et al. (1999) Vaccine 17: 2927-2938), can also be used as a carrier for a composition of the invention.

Pharmaceutically acceptable salts can also be used in compositions of the invention, for example, mineral salts such as hydrochlorides, hydrobromides, phosphates, or sulfates, as well as salts of organic acids such as acetates, proprionates, malonates, or benzoates.

5 Compositions of the invention generally contain pharmaceutically acceptable excipients, such as water, saline, glycerol, and ethanol, as well as substances such as wetting agents, emulsifying agents, or pH buffering agents.

 The chimeric molecules of the present invention may be used for nucleic acid immunization, to generate an appropriate immune response, such as to activate HCV-
10 specific T cells, using standard gene delivery protocols. Any method known in the art can be employed to package and deliver nucleic acid molecules of the invention, including nucleic acid molecules in an immunogenic composition. Methods for gene delivery are known in the art. See, e.g., U.S. Patent Nos. 5,399,346, 5,580,859, 5,589,466, incorporated by reference herein in their entireties. For example, the coding
15 sequences can be packaged in a viral vector, combined with lipid, peptoid excipients, PLG formulations, or gold particles. Preferably, an immunogenic composition is delivered as naked DNA or naked RNA. The expressed immunogenic polypeptide is preferably presented to the host immune system with native post-translational modifications, structure, and conformation.

20 For example, the constructs can be packaged in liposomes prior to delivery to the cells. Lipid encapsulation is generally accomplished using liposomes which are able to stably bind or entrap and retain nucleic acid. The ratio of condensed DNA to lipid preparation can vary but will generally be around 1:1 (mg DNA:micromoles lipid), or more of lipid. For a review of the use of liposomes as carriers for delivery of nucleic
25 acids, see, Hug and Sleight, *Biochim. Biophys. Acta.* (1991) 1097:1-17; Straubinger et al., in *Methods of Enzymology* (1983), Vol. 101, pp. 512-527.

 Liposomal preparations for use with the present invention include cationic (positively charged), anionic (negatively charged) and neutral preparations, with cationic liposomes particularly preferred. Cationic liposomes are readily available. For example,
30 N[1-2,3-dioleoyloxy)propyl]-N,N,N-triethylammonium (DOTMA) liposomes are available

under the trademark Lipofectin, from GIBCO BRL, Grand Island, NY. (See, also, Felgner et al., *Proc. Natl. Acad. Sci. USA* (1987) 84:7413-7416). Other commercially available lipids include transfectace (DDAB/DOPE) and DOTAP/DOPE (Boehringer). Other cationic liposomes can be prepared from readily available materials using techniques well known in the art. See, e.g., Szoka et al., *Proc. Natl. Acad. Sci. USA* (1978) 75:4194-4198; PCT Publication No. WO 90/11092 for a description of the synthesis of DOTAP (1,2-bis(oleoyloxy)-3-(trimethylammonio)propane) liposomes. The various liposome-nucleic acid complexes are prepared using methods known in the art. See, e.g., Straubinger et al., in *METHODS OF IMMUNOLOGY* (1983), Vol. 101, pp. 512-527; Szoka et al., *Proc. Natl. Acad. Sci. USA* (1978) 75:4194-4198; Papahadjopoulos et al., *Biochim. Biophys. Acta* (1975) 394:483; Wilson et al., *Cell* (1979) 17:77; Deamer and Bangham, *Biochim. Biophys. Acta* (1976) 443:629; Ostro et al., *Biochem. Biophys. Res. Commun.* (1977) 76:836; Fraley et al., *Proc. Natl. Acad. Sci. USA* (1979) 76:3348; Enoch and Strittmatter, *Proc. Natl. Acad. Sci. USA* (1979) 76:145; Fraley et al., *J. Biol. Chem.* (1980) 255:10431; Szoka and Papahadjopoulos, *Proc. Natl. Acad. Sci. USA* (1978) 75:145; and Schaefer-Ridder et al., *Science* (1982) 215:166.

The DNA can also be delivered in cochleate lipid compositions similar to those described by Papahadjopoulos et al., *Biochem. Biophys. Acta.* (1975) 394:483-491. See, also, U.S. Patent Nos. 4,663,161 and 4,871,488.

A number of viral based systems have been developed for gene transfer into mammalian cells. For example, retroviruses provide a convenient platform for gene delivery systems, such as murine sarcoma virus, mouse mammary tumor virus, Moloney murine leukemia virus, and Rous sarcoma virus. A selected gene can be inserted into a vector and packaged in retroviral particles using techniques known in the art. The recombinant virus can then be isolated and delivered to cells of the subject either *in vivo* or *ex vivo*. A number of retroviral systems have been described (U.S. Patent No. 5,219,740; Miller and Rosman, *BioTechniques* (1989) 7:980-990; Miller, A.D., *Human Gene Therapy* (1990) 1:5-14; Scarpa et al., *Virology* (1991) 180:849-852; Burns et al., *Proc. Natl. Acad. Sci. USA* (1993) 90:8033-8037; and Boris-Lawrie and Temin, *Cur. Opin. Genet. Develop.* (1993) 3:102-109. Briefly, retroviral gene delivery vehicles of the

present invention may be readily constructed from a wide variety of retroviruses, including for example, B, C, and D type retroviruses as well as spumaviruses and lentiviruses such as FIV, HIV, HIV-1, HIV-2 and SIV (see RNA Tumor Viruses, Second Edition, Cold Spring Harbor Laboratory, 1985). Such retroviruses may be readily
5 obtained from depositories or collections such as the American Type Culture Collection ("ATCC"; 10801 University Blvd., Manassas, VA 20110-2209), or isolated from known sources using commonly available techniques.

A number of adenovirus vectors have also been described, such as adenovirus Type 2 and Type 5 vectors. Unlike retroviruses which integrate into the host genome,
10 adenoviruses persist extrachromosomally thus minimizing the risks associated with insertional mutagenesis (Haj-Ahmad and Graham, *J. Virol.* (1986) 57:267-274; Bett et al., *J. Virol.* (1993) 67:5911-5921; Mittereder et al., *Human Gene Therapy* (1994) 5:717-729; Seth et al., *J. Virol.* (1994) 68:933-940; Barr et al., *Gene Therapy* (1994) 1:51-58; Berkner, K.L. *BioTechniques* (1988) 6:616-629; and Rich et al., *Human Gene Therapy*
15 (1993) 4:461-476).

Molecular conjugate vectors, such as the adenovirus chimeric vectors described in Michael et al., *J. Biol. Chem.* (1993) 268:6866-6869 and Wagner et al., *Proc. Natl. Acad. Sci. USA* (1992) 89:6099-6103, can also be used for gene delivery.

Members of the Alphavirus genus, such as but not limited to vectors derived from
20 the Sindbis and Semliki Forest viruses, VEE, will also find use as viral vectors for delivering the gene of interest. For a description of Sindbis-virus derived vectors useful for the practice of the instant methods, see, Dubensky et al., *J. Virol.* (1996) 70:508-519; and International Publication Nos. WO 95/07995 and WO 96/17072.

Other vectors can be used, including but not limited to simian virus 40,
25 cytomegalovirus. Bacterial vectors, such as Salmonella ssp. *Yersinia enterocolitica*, Shigella spp., *Vibrio cholerae*, Mycobacterium strain BCG, and *Listeria monocytogenes* can be used. Minichromosomes such as MC and MC1, bacteriophages, cosmids (plasmids into which phage lambda *cos* sites have been inserted) and replicons (genetic elements that are capable of replication under their own control in a cell) can also be
30 used.

The chimeric constructs may also be encapsulated, adsorbed to, or associated with, particulate carriers. Such carriers present multiple copies of a selected molecule to the immune system and promote trapping and retention of molecules in local lymph nodes. The particles can be phagocytosed by macrophages and can enhance antigen presentation through cytokine release. Examples of particulate carriers include those
5 derived from polymethyl methacrylate polymers, as well as microparticles derived from poly(lactides) and poly(lactide-co-glycolides), known as PLG. See, e.g., Jeffery et al., *Pharm. Res.* (1993) 10:362-368; and McGee et al., *J. Microencap.* (1996).

A wide variety of other methods can be used to deliver the constructs to cells.
10 Such methods include DEAE dextran-mediated transfection, calcium phosphate precipitation, polylysine- or polyornithine-mediated transfection, or precipitation using other insoluble inorganic salts, such as strontium phosphate, aluminum silicates including bentonite and kaolin, chromic oxide, magnesium silicate, talc, and the like. Other useful methods of transfection include electroporation, sonoporation, protoplast fusion,
15 liposomes, peptoid delivery, or microinjection. See, e.g., Sambrook et al., *supra*, for a discussion of techniques for transforming cells of interest; and Felgner, P.L., *Advanced Drug Delivery Reviews* (1990) 5:163-187, for a review of delivery systems useful for gene transfer. One particularly effective method of delivering DNA using electroporation is described in International Publication No. WO/0045823.

20 Additionally, biolistic delivery systems employing particulate carriers such as gold and tungsten, are useful for delivering the constructs of the present invention. The particles are coated with the construct to be delivered and accelerated to high velocity, generally under a reduced atmosphere, using a gun powder discharge from a "gene gun." For a description of such techniques, and apparatuses useful therefore, see, e.g., U.S.
25 Patent Nos. 4,945,050; 5,036,006; 5,100,792; 5,179,022; 5,371,015; and 5,478,744.

The chimeric constructs can be delivered either directly to the vertebrate subject or, alternatively, delivered *ex vivo*, to cells derived from the subject and the cells reimplanted in the subject. For example, the constructs can be delivered as plasmid DNA, e.g., contained within a plasmid, such as pBR322, pUC, or ColE1.

An immunogenic composition of the invention is administered in a manner compatible with the particular composition used and in an amount which is effective to elicit an anti-HCV polypeptide antibody titer, such as an anti-E2 or anti-E1 antibody titer. Administration can be by any means known in the art, including intramuscular, 5 intradermal, intraperitoneal, or subcutaneous injection, including injection using a biological ballistic gun ("gene gun"). Electroporation or iontophoresis can also be used. Administration may also be intranasal or oral. For oral administration of an immunogenic composition, a protein carrier is preferably included. An immunogenic composition, including compositions comprising naked DNA or RNA, is preferably 10 injected intramuscularly to a large mammal, such as a baboon, chimpanzee, or human, at a dose of 0.5, 0.75, 1.0, 1.5, 2.0, 2.5, 5 or 10 mg/kg. A composition comprising virus-like particles is preferably injected intramuscularly to a large mammal, such as a human, at a dose of 0.01, 0.05, 0.5, 0.75, 1.0, 1.5, 2.0, 2.5, 5 or 10 mg protein/kg body weight.

Direct delivery of the compositions will generally be accomplished by injection, 15 either subcutaneously, intraperitoneally, intravenously or intramuscularly or delivered to the interstitial space of a tissue. The compositions can also be administered into a lesion. Other modes of administration include oral, intranasal, and pulmonary administration, suppositories, and transdermal or transcutaneous applications (*e.g.*, see WO98/20734), needles, and gene guns or hyposprays. Dosage treatment may be a single dose schedule 20 or a multiple dose schedule. The composition may be administered in conjunction with other immunoregulatory agents.

Methods for the *ex vivo* delivery and reimplantation of transformed cells into a subject are known in the art and described in *e.g.*, WO93/14778. Examples of cells useful in *ex vivo* applications include, for example, stem cells, particularly hematopoietic, 25 lymph cells, macrophages, dendritic cells, or tumor cells. Generally, delivery of nucleic acids for both *ex vivo* and *in vitro* applications can be accomplished by the following procedures, for example, dextran-mediated transfection, calcium phosphate precipitation, polybrene mediated transfection, protoplast fusion, electroporation, encapsulation of the polynucleotide(s) in liposomes, and direct microinjection of the DNA into nuclei, all well 30 known in the art.

Immunogenic compositions comprising either HBV/HCV virus-like particles or nucleic acids encoding and expressing such virus-like particles can be administered either to a mammal that is not infected with HCV or can be administered to an HCV-infected mammal. The particular dosages of virus-like particles or nucleic acids will depend on a number of factors including, but not limited to, the species, age, and general condition of the mammal to which the composition is administered and the mode of administration of the composition. An effective amount of the composition of the invention can be readily determined using routine experimentation. *In vivo* models are well known and can be employed to identify appropriate doses. If desired, co-stimulatory molecules or adjuvants can also be provided before, after, or together with the immunogenic compositions. Immunogenic compositions can be administered more than once, as required for effective immunization. Administration of immunogenic compositions containing virus-like particles can be performed either before or after administration of immunogenic compositions containing nucleic acids, such that one form (protein or nucleic acid) provides a primary immunization and the other form boosts the immune response.

III. Experimental

Below are examples of specific embodiments for carrying out the present invention. The examples are offered for illustrative purposes only, and are not intended to limit the scope of the present invention in any way. Those of skill in the art will readily appreciate that the invention may be practiced in a variety of ways given the teaching of this disclosure.

Efforts have been made to ensure accuracy with respect to numbers used (e.g., amounts, temperatures, etc.), but some experimental error and deviation should, of course, be allowed for.

EXAMPLE 1

Detection of HBV Virus-Like Particles Using Sucrose Density Gradient Sedimentation

Purified recombinant HBsAg particles were obtained in the form of a hepatitis B vaccine from Chiron Corp. Clinical Department. Two hundred fifty μL of a 37 $\mu\text{g}/\text{mL}$ suspension in 0.03 M citrate, 0.26 M NaCl, 0.005% polysorbate 80 was loaded onto a 5-30% (wt-vol) sucrose gradient and centrifuged 4 hrs at 40,000 rpm in a Beckman SW41 rotor. Ten fractions were removed and assayed for HBsAg by the Magic Lite Assay. The results are shown in Fig. 5.

Magic Lite Assay

This describes a highly sensitive immunochemiluminescence assay used to detect HBV and HCV antigens. One hundred μL of sample was added to a 12 x 75 mm polystyrene tube. One hundred μL (30 μg) of a "capture" antibody was added to the sample. The capture antibody was covalently linked to paramagnetic particles by glutaraldehyde crosslinking a mixture of 6:1 paramagnetic particles:antibody. The tubes were vortexed and incubated in a 37°C waterbath for 20 minutes. Next the tubes were placed on a magnetic rack where the paramagnetic particles (PMP) can aggregate. This allowed for washing and decanting. After the third wash, 100 μL of a "detecting" antibody, which was covalently linked to dimethylacridinium, was added and the tube was vortexed. The tubes were again incubated at 37°C for 20 minutes and then placed on a magnetic rack for washing and decanting. After the third wash, the tubes were placed in a Ciba Corning Magic Lite chemiluminometer to measure the analyte bound. Results were expressed in arbitrary units of light intensity (relative light units, RLU).

Antibodies

Anti-sAg was provided by Chiron Diagnostics (Walpole, MA) and was directed against serotypes AYW and ADW. MAbs 5E5/H7 was obtained from mice using HeLa E1/E2 amino acids 1-967 as an immunogen and recognizes the E2 epitope 384-655. MAbs 3D5/C3 was also obtained from mice immunized with HeLa E1/E2 amino acids 1-967 and recognizes E1 epitope 211-217.

EXAMPLE 2

Expression of HBV/HCV Virus-Like Particles Containing

Chimeric HBsAg-E2-661 Antigen in COS7 Cells

Five μ g of pCMV-II-E2661-sAg (Fig. 4A) and increasing amounts of pCMV-II-pS2-sAg (Fig. 2A) were prepared. Expression from pCMV-II-pS2-sAg resulted in a mixture of about 5-20% preS2-S-polypeptide, with the remainder being S-polypeptide. Ratios of sAg to E2661-sAg plasmids used were 0:1, 1:1, 5:1, and 10:1 on a μ g DNA basis. The total amount of DNA in each tube was normalized to 55 μ g by the addition of pCMV-km-Bgal. This mixture was transfected into COS7 cells using the LT1 transfection reagent from Panvera, as described below. At 48 hours post-transfection, media and soluble lysates (obtained by incubating cell monolayers in PBS with 0.1% NP40 and centrifuging to remove insoluble debris) were removed and assayed by the Magic Lite Assay, with capture by anti-sAg followed by detection with a conjugated anti-E2 MAAb (5E5/H7, see Example 1) (Fig. 6A) or MAAb sAg (Fig. 6B). Both E2 and sAg were increasingly secreted into the medium as the ratio of sAg:E2661-sAg was increased, with an optimum secretion at ratios in the range of 5:1 to 10:1.

In order to characterize the virus-like particles, 1 mL of culture medium from each condition was loaded onto a 5-30% sucrose gradient. The samples were centrifuged 4 hrs at 40,000 rpm using a Beckman SW41 rotor. Eleven fractions were removed and assayed for E2 (Fig. 7A) or sAg (Fig. 7B) by the Magic Lite Assay. Both E2 and sAg were observed in highest amount in fraction 4, which is the same gradient position for the peak distribution of HBsAg-containing virus-like particles (Fig. 5).

Transfection Protocol

Cells were seeded in 6-well plates the day prior to transfection so as to achieve 50-60% confluency at the time of transfection. Opti-mem (100 μ L) and LT-1 transfection reagent (12 μ L) were added to a sterile polypropylene tube and incubated at room temperature for five minutes. Three μ g of DNA was then added to the tube, which was incubated another five minutes at room temperature. During this incubation period, the cells were washed with Opti-mem (2 mLs per well). Two mLs Opti-mem were added

to each well, followed by 100 μ L of the Opti-mem/LT-1/DNA mixture. The cells were then incubated for four hours at 37°C, followed by aspiration and addition of culture medium (1.5 mL/well of DMEM + 10% FBS). At 48 hours post transfection, the medium was harvested and the cell monolayers were solubilized using PBS containing 0.1% NP-40. Both the harvested medium and the solubilized cells were centrifuged to remove debris.

EXAMPLE 3

Expression of HBV/HCV Virus-Like Particles Containing Chimeric HBsAg-E1-330 Antigen in COS7 Cells

Mixtures of pCMV-II-optiE1330-sAg (2.5 μ g DNA) and various amounts of pCMV-II-pS2-sAg were prepared to provide ratios of sAg plasmid to E1-sAg plasmid of 0:1 (control), 1:1, 5:1, 10:1, and 20:1. Expression from pCMV-II-pS2-sAg resulted in a mixture of about 5-20% preS2-S-polypeptide, with the remainder being S-polypeptide. The total amount of DNA in each tube was normalized to 52.5 μ g by the addition of pCMV-km- β gal. The mixtures were transfected into COS7 cells using the LT1 transfection reagent from Panvera. At 48 hours post-transfection, the media and soluble lysates were recovered and assayed by the Magic Lite Assay. Capture was with anti-sAg followed by detection with a conjugated anti-E1 MAbs (eD5/C3) (Fig. 8A) or MAbs sAg (Fig. 8B). Both E1 and sAg were increasingly secreted into the medium as the ratio of sAg:E2661-sAg was increased, with an optimum secretion at ratios in the range of 5:1 to 20:1.

In order to characterize the virus-like particles, 1 mL of culture medium from each condition was loaded onto a 5-30% sucrose gradient. The samples were centrifuged 4 hrs at 40,000 rpm using a Beckman SW41 rotor. Eleven fractions were removed and assayed for E1 (Fig. 9A) or sAg (Fig. 9B) by the Magic Lite Assay. Both E1 and sAg were observed in highest amount in fractions 4-6, which is similar to the gradient position for the peak distribution of HBsAg-containing virus-like particles (Fig. 5).

EXAMPLE 4

Expression of HBV/HCV Virus-Like Particles Containing Both

HBsAg-E2-661 and HBsAg-E1-330 Chimeric Antigens in COS7 Cells

A mixture of pCMVII opti 330 E1-sAg and pCMV-II-E2661-sAg (0.5 µg DNA of
5 each plasmid) was combined with increasing amounts of pCMV-II-pS2-sAg. The total
amount of DNA in each tube was normalized to 26 µg by the addition of pCMV-kM-
βgal. The mixture was transfected into COS7 cells using the LT1 transfection reagent
from Panvera. At 48 hours post-transfection, media and soluble lysates were recovered
and assayed by the Magic Lite Assay, with capture by anti-sAg followed by detection
10 with a conjugated anti-E2 or anti-E1 MAb (Fig. 10A) or a conjugated anti-sAg (Fig.
10B).

EXAMPLE 5

Use of HBV/HCV Virus-Like Particles to Generate an Immune Response

In order to test the ability of the subject HBV/HCV virus-like particles to generate
15 an immune response *in vivo*, the following experiment was done. Four groups of 10 mice
(Group 2 had 9 mice) were administered either DNA encoding HCV E2661 and pCMV-
II, at a ratio of 5 times pCMV-II to E2661 (Group 1); pCMV-II-E2661-sAg (Fig. 4A) and
pCMV-II, at a ratio of 5 times pCMV-II to pCMV-II-E2661-sAg (Group 2); pCMV-II-
E2661-sAg and pCMV-II-pS2-sAg (Fig. 2A) at a ratio of 5 times pCMV-II-pS2-sAg to
20 pCMV-II-E2661-sAg (Group 3); and pCMV-II and pCMV-II-pS2-sAg, at a ratio of 5
times pCMV-II-pS2-sAg to pCMV-II (Group 4).

All were immunized twice with 90 µg (45 µg/leg) per immunization, at day 0 and
day 21, and blood was collected. Antibody titers were assessed by ELISA using a
truncated E2 molecule, E2₇₁₅, which had been expressed in CHO cells, using the Magic
25 Lite Assay described above. Results are shown in Table 1.

Group #	Treatment	GM +/- SE
1	E2661 + 5x vector	1300 +/- 85
2	E2661-sAg + 5x vector	1547 +/- 690
3	E2661-sAg + 5x pS2sAg	4175 +/- 603
4	Vector + 5x pS2sAg	0

A second experiment was run, again using 10 animals per group, except Group 5, which had 5 mice. The groups and results are shown in Table 2. Treatment protocol and antibody determination was as described above.

Group #	Treatment	GM +/- SE
1	E2661 + 5x vector	724 +/- 96
2	E2661 + 5x pS2sAg	222 +/- 90
3	E2661-sAg + 5x vector	1018 +/- 305
4	E2661-sAg + 5x pS2sAg	1290 +/- 292
5	mock	0

As can be seen, titers to E2 in the E2₆₆₁-immunized mice were reduced in the presence of 5x sAg. This might have resulted from competition for docking to the endoplasmic reticulum. The E2 titers were higher when the E2 was fused to sAg. The addition of sAg in place of vector resulted in little change in titer. While this difference is small, comparison to the control with sAg is warranted. Since excess sAg caused a reduction in E2 titers in the unfused antigen, in the context of the fusion there was an apparent compensation. This presumably reflects the ability of sAg to promote secretion of the fusion.

Thus, chimeric HCV/HBV virus-like particles, as well as methods of making and using the same are disclosed. From the foregoing, it will be appreciated that, although specific embodiments of the invention have been described herein for purposes of illustration, various modifications may be made without deviating from the spirit and scope of the appended claims.